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Identification and Quantification of Polybrominated Hexahydroxanthene Derivatives and Other Halogenated Natural Products in Commercial Fish and Other Marine Samples

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During routine analysis of commercial fish on halogenated pollutants, an unknown tribromo component (TriBHD) was initially detected as an abundant peak in sample extracts from the Mediterranean Sea. The molecular formula was established to be $C_{16}H_{19}Br_3O$ by gas chromatography with electron ionization high-resolution mass spectrometry (GC/EI-HRMS). GC/EI-MS data were virtually identical with a polybrominated hexahydroxanthene derivative (PBHD) previously isolated from an Australian sponge species known to occur in the Mediterranean Sea as well. A tetrabromo isomer (TetraBHD) was also found in the fish samples. The concentrations of TriBHD and other halogenated compounds in commercial fish (sea bass, gilt head bream, anchovy, sardine, and salmon) were estimated with GC/electron capture detection (ECD). Using the ECD response of *trans*-nonachlor, the concentration of TriBHD reached up to 90 ng/g lipid weight and accounted for up to >90% of the concentration of *p*,*p*′-DDE, which was the most abundant peak in the most samples investigated. On the basis of the GC/ECD response, TetraBHD amounted for ~1/7 of TriBHD in all fish samples investigated. The sample with the highest content was a green-lipped mussel from New Zealand (236 ng/g lipid weight). The halogenated natural products TBA, Q1, and MHC-1 were also present in most of the samples. We assume that the bulk of the residues in fish from aquaculture may originate from algae and sponges living in proximity of the fish farms. Detection of TriBHD and TetraBHD in blubber of a monk seal (*Monachus monachus*) suggests that both HNPs may reach the top predators of food webs and thus also humans.

KEYWORDS: Brominated xanthene derivates; naturally occurring organohalogens; commercial fish; sponge metabolites; pollutants; GC-MS

INTRODUCTION

Organohalogen compounds are serious contaminants of food and the environment. The permanent control of substances such as PCBs, DDT, and other chloropesticides is an important task in consumer protection. To the compounds mentioned above, several other classes of organohalogen compounds have been added (*1*). These include polybrominated flame retardants, whose concentrations are increasing in several regions of the world (*2*). In addition, there are several publications discussing organohalogen compounds of natural origin, which have been detected in top predators of marine food webs, i.e., seabirds and marine mammals (*3*−*9*). These halogenated natural products (HNPs) are secondary metabolites of a huge variety of marine organisms (*10*, *11*). More than 4000 HNPs have been detected in marine seaweed, sponges, bacteria, and other organisms (*12*, *13*). However, only a few of them including TBA, 2,3,3′,4,4′,5,5′-heptachloro-1′-methyl-1,2′-bipyrrole (Q1), and MHC-1 have been detected in higher organisms (**Figure 1**). During routine analysis in German food control, an abundant tribromo compound was detected as an abundant peak in the gas chromatography/electron capture detection (GC/ECD) chromatograms of commercial fish from the Mediterranean Sea. The compound did not match the retention time of any known brominated pollutant. In this study, we provide evidence that the tribromo compound and a tetrabromo congener are HNPs most likely originating from marine sponges.

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MATERIAL AND METHODS

Chemicals. The purity of solvents used for fish analysis (*n*-hexane, acetone, cyclohexane, ethyl acetate, isooctane, and toluene) was "zur Rückstandsanalyse" (Promochem, Wesel, Germany). PCB and pesticide

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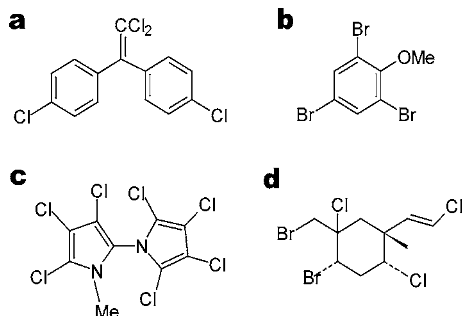

Figure 1. Structures of (**a**) the key metabolite of man-made DDT in biota (*p,p′*-DDE) and the HNPs (**b**) TBA, (**c**) Q1, and (**d**) one of the potential structures of MHC-1 (*6*).

reference standards were from Dr. Ehrenstorfer (Augsburg, Germany). Sodium sulfate and sea sand were heated for at least 5 h at 650 °C. Silica (Kieselgel 60, 70−230 mesh; Merck, Germany) was activated for 5 h at 130 °C. The origin and quality of solvents and chemicals used for analysis of marine mammals were published in detail elsewhere (*14*).

Samples. All fish samples were taken as part of the official food control in Bavaria (Germany) from local retailers and wholesalers. Under these routine inspections, the edible part of seafood was analyzed for residues of pesticides and PCBs. Because of the frequent detection of 2,7-dibromo-4a-bromomethyl-1,1-dimethyl-2,3,4,4a,9,9a-hexahydro-1*H*-xanthene (TriBHD) and 2,5,7-tribromo-4a-bromomethyl-1,1-dimethyl-2,3,4,4a,9,9a-hexahydro-1*H*-xanthene (TetraBHD) in fish from the Mediterranean Sea, we also investigated a sample of monk seal (*Monachus monachus*) that died at the Western Saharan coast of Africa (Mauritania) in 1997 (*15*). Fresh green-lipped mussels (*Perna canaliculus*) were from the local market in Bavaria, whereas powder (0.5 g units in gelatin) was ordered from Southern Deep at www.lovelyhealth.com.

Sample Cleanup. Twenty grams of fish fillet was ground with water-free Na_2SO_4 (∼250 g) and sea sand (∼50 g) to obtain dry and homogeneous material. Column extraction of lipophilic compounds was performed with 300 mL of *n*-hexane/acetone (2:1, v/v) according to Ernst et al. (*16*). A 0.5 g amount of the resulting fish oil and 50 ng of isodrin (internal standard) were dissolved in 5 mL of ethyl acetate/cyclohexane (1:1, v:v), and 4 mL was subjected to gel permeation chromatography (GPC) with bio-beads S-X3 and elution with ethyl acetate/cyclohexane (1:1, v:v). After GPC, the solvent was changed to isooctane and concentrated to 1 mL. The isooctane extract was either adjusted to 4 mL and treated with 2 mL of concentrated sulfuric acid or purified on 1 g of deactivated silica (1.5% water, w:v). Elution with 8 mL of *n*-hexane provided a solution with PCBs and other, mainly aromatic, organohalogen compounds (fraction A). Subsequent elution with 8 mL of *n*-hexane/toluene (63:35, v:v) yielded fraction B, which included more polar organohalogen compounds (e.g., nonaromatic chloropesticides and polybrominated compounds). The fractions were concentrated to 4 mL and analyzed by GC/ECD. Sample cleanup was performed according to the official German procedure, developed for the determination of chloropesticides and PCBs in fish (*17*). For GC/electron ionization−mass spectrometry (EI-MS) investigation, we used an enriched solution obtained by combination and concentration of sample extracts. Sample cleanup of the monk seal sample and the mussel powder was described elsewhere (*14*, *18*).

GC in Combination with ECD. Fish sample extracts were analyzed with a GC/ECD system consisting of a Hewlett-Packard 6890 gas chromatograph equipped with an autosampler and a split/splitless injector. He (5.0 quality) was used as a carrier gas at a constant pressure of 1.5 bar. Ar/CH_4 (90/10) was used as a makeup gas at a flow rate of 40 mL/min. Two microliters was injected (splitless time, 1 min) at 285 °C. A t-piece after the injector in the oven divided the gas flow onto two capillary columns, which both ended in ECDs operated at 300 °C. The 30 m × 0.25 mm i.d. capillary columns were coated with 0.25 μm 95% dimethyl, 5% methylphenyl polysiloxane (HP-5), or 100% dimethyl polysiloxane (HP-1). The GC oven program was arranged as follows. After 2 min at 90 °C, the temperature was raised at 30 °C/min to 150 °C, then at 3 °C/min to 204 °C (hold time, 3 min), and finally at 8 °C/min to 280 °C (hold time, 10 min). The run time was 44.5 min. Quantification of TriBHD, TetraBHD, MHC-1, and Q1 in fish was carried out relative to the peak area of the external standard *trans*-nonachlor (*18*). In dependence of the sample weight and final volume of purified extracts, the limit of detection was typically in the range of 0.3−1 μg/kg lipids.

GC in Combination with MS. Low-resolution mass spectrometric analyses were performed with a Varian 3800 gas chromatograph interfaced to a Varian 1200 triple quad mass spectrometer. A 30 m × 0.25 mm i.d. capillary column coated with 0.25 μm d_f Factor Four CP-Sil 8ms (Varian, Darmstadt, Germany) was installed in the GC oven. The GC oven was heated with the following temperature program: 70 °C (1.5 min), 30 °C/min to 140 °C, 3 °C/min to 230 °C (25 min), and 4 °C/min to 270 °C (36 min). The injector and the transfer line temperatures were set at 250 and 280 °C, respectively. Injections were performed in splitless mode (split opened after 2 min), and a constant flow rate of 1.0 mL/min He (purity 5.0) was used throughout the measurement. In the GC/EI-MS mode, a mass range from *m/z* 200 to 650 was chosen for the recording of full scan spectra. The scan time was set at 0.5 s/cycle. In the GC/ECNI-MS mode, methane (purity 4.5) was used as the reagent gas at a pressure of 8 Torr in the ion source. In the selected ion monitoring mode, *m/z* 79, 81, 159, and 161 were measured throughout the run. In the full scan mode, *m/z* 70−550 was scanned twice a second.

Additional GC/EI-MS analyses were performed with a Hewlett-Packard 6890 gas chromatograph coupled to a 5973 MSD, and some GC/ECNI-MS analyses were performed with a Hewlett-Packard 5980 series II gas chromatograph interfaced to a 5989B mass spectrometer using the parameters previously described in detail (*6*).

GC in Combination with EI−High-Resolution Mass Spectrometry (HRMS). Analyses was performed on an Autospec MasSpec (Micromass, Manchester, United Kingdom) double-focusing magnetic sector mass spectrometer (geometry EBE) connected to a Hewlett-Packard 6890N gas chromatograph, equipped with a DB-5 (J&W Scientific) nonpolar capillary column (30 m × 0.25 mm i.d. × 0.25 μm d_f). The GC injection port and transfer line temperatures were set at 280 °C, and the samples (1 μL) were injected in the splitless mode. The oven temperature program started at 70 °C (2 min isothermal), which then was raised at 25 °C/min to 150 °C, at 3 °C/min to 200 °C, and at 8 °C/min to 280 °C (10 min isothermal). The carrier gas (He) flow was 1 mL/min. Mass spectra were measured in the EI mode at 38 eV, with a source temperature of 265 °C and an acceleration voltage of 8 kV. For full scan measurements, the instrument was scanned 100 u around the ion of interest at 10 s/decade and at a resolution of 8000 (5% valley). Perfluorokerosene (PFK, high boiling, Fluka) was used as a calibration gas.

RESULTS AND DISCUSSION

Figure 2 shows the GC/ECD chromatogram of the entire organohalogen fraction of a farmed Mediterranean fish sample, which contained TriBHD as the second most abundant peak after *p,p′*-DDE. TriBHD eluted after the pentabromodiphenyl ether congener BDE 99 (not present in the sample) from nonpolar GC stationary phases. Next to *p,p′*-DDD, the HNPs 2,4,6-tribromoanisole (TBA) and MHC-1 caused abundant, more relevant peaks than the key PCBs in fish, i.e., PCB 153 and PCB 138 (coeluted with PCB 163) (**Figure 1**). During standard sample cleanup (see Materials and Methods), TriBHD eluted into fraction B typically of chloropesticides (HCH, toxaphene, and chlordane) and polybrominated compounds (PBDEs, PBBs, and BCs). However, the retention time of TriBHD did not agree with that of any known pollutant in fish samples. Interestingly, a second, lower abundant peak (TetraBHD, **Figure 2**) was exclusively present in samples containing TriBHD. Therefore, we paid attention to this compound as well (see below).

The ions at *m/z* 79 and 81 in the GC/ECNI-MS full scan spectrum of TriBHD unequivocally prove the presence of

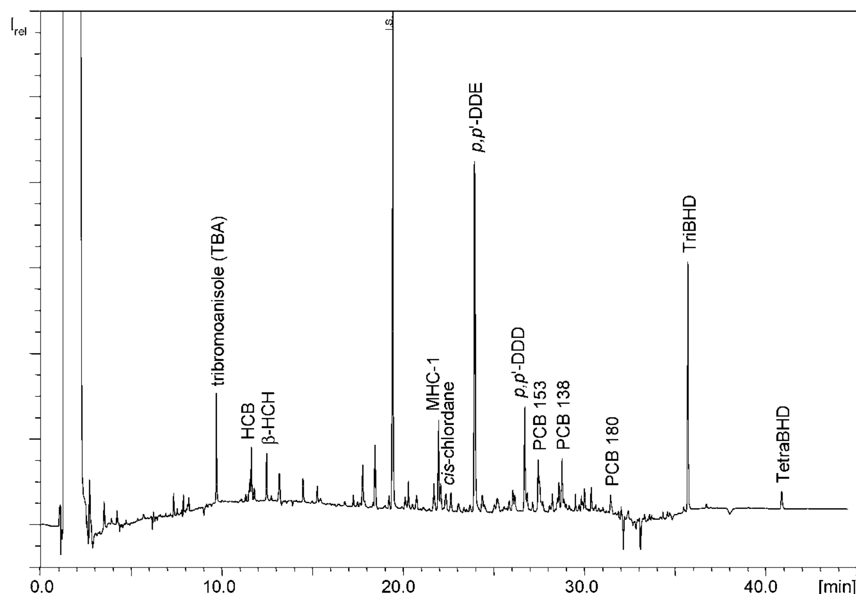

Figure 2. GC/ECD chromatogram (HP-1) of sea bass (complete organohalogen fraction) from the Mediterranean Sea.

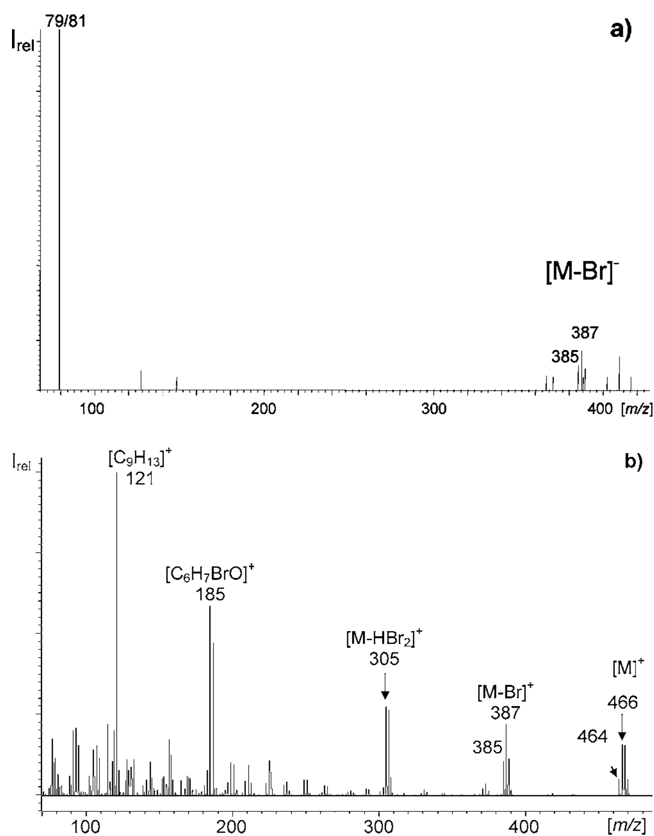

Figure 3. (**a**) GC/ECNI-MS and (**b**) GC/EI-MS full-scan mass spectrum of TriBHD as determined in commercial fish.

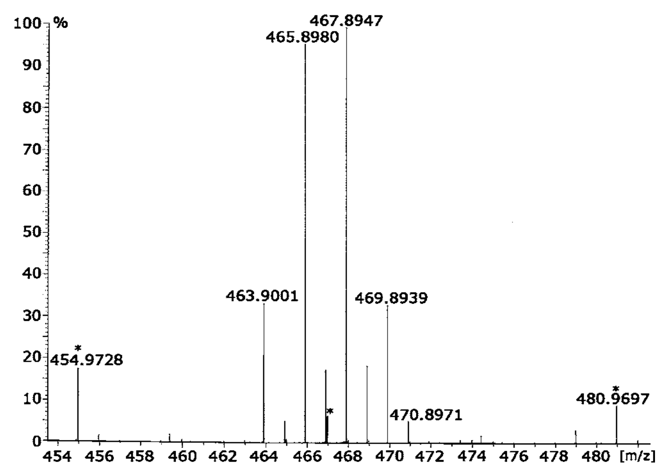

Figure 4. GC/HR-EIMS analysis of the molecular ion of TriBHD in a sample extract of fish. * labeled masses originate from PFK.

bromine substituents (**Figure 3a**). No other fragment ion except the dibromo fragment ion at *m/z* 385 was detected in the GC/ECNI-MS full scan run. The absence of the $[HBr_2]^-$ fragment ion at *m/z* 159 clarified that TriBHD is not a brominated diphenyl ether derivative (*14*). Furthermore, *m/z* 385 (Br_2 compound) is not found in GC/ECNI-MS spectra of polybrominated biphenyls. This fragment ion requires either an odd number of nitrogen on the molecule or that *m/z* 385 is not the molecular ion. A concentrated sample was thus studied by GC/EI-MS in the full scan mode (**Figure 3b**). The GC/EIMS also showed an intense fragment ion at *m/z* 385, but the tribromo isotope pattern at *m/z* 464 verified three bromine substituents on the compound. Monobromo fragment ions at *m/z* 305 ($[M - Br - HBr]^+$), *m/z* 306 ($[M - 2\ Br]^+$), and *m/z* 185 were detected as well. However, the base peak at *m/z* 121 originated from a nonbrominated fragment ion. This mass spectrometric feature along with a relatively low molecular mass for the observed retention time suggested a rather unconventional backbone for TriBHD. This is remarkable since the molecular mass of *m/z* 464 would have been met by an unknown tribromo isomer of the HNP dimethoxytetrabromophenoxyanisole BC-11 ($C_{14}H_{10}Br_4O_3$, *m/z* $542 - 79 + 1 = C_{14}H_{11}Br_3O_3$, *m/z* 464). To establish the molecular formula of TriBHD, we performed selected experiments using GC/EI-HRMS. The shape of the molecular ion (**Figure 4**) is in perfect agreement with the presence of three bromine substituents on TriBHD. The exact mass of the major abundant isotopic peak was determined to be 467.8947, which is only 0.2 mu or 0.4 ppm higher than the theoretical value of $C_{16}H_{19}Br_3O$. The lower abundant monoisotopic peak differed by 1.5 mu or 3.2 ppm from the theroretical value of 463.898599 but still are in the range of positive identification. By contrast, the tribromo congener of BC-11 ($C_{14}H_{11}Br_3O_3 = 168 + 11.086075 + 236.755044 + 47.984745$) would have been 463.825864 or 70 mu different from the measured value. The good resolution from the PFK mass *m/z*

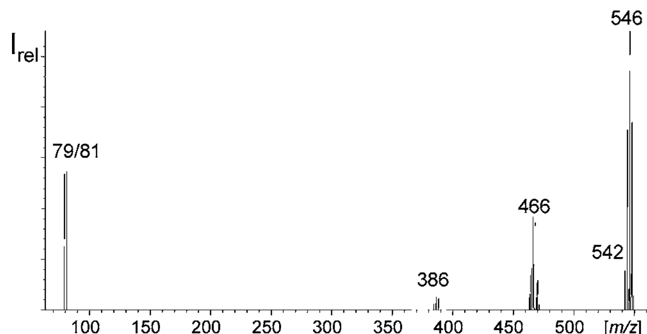

Figure 5. GC/ECNI-MS of TetraBHD in a sample of monk seal (*M. monachus*) blubber from Mauretania.

466.9728 confirmed the very good and reliable measurement. **Figure 4** also allows estimatation of the number of carbons on TriBHD. On the basis of the major isotope peak (=100%), the ^{13}C satellite peak accounted for 18% of the ^{12}C signal. This is given for 16.4 carbons, which matches the molecular formula derived from the HRMS determination. No other elemental composition with 14−18 C, three Br, any numbers of O and H, and equal numbers of N could be calculated within 463.8985 ± 0.01. Further fragment ions were investigated as well by HRMS (see below) and fully supported the measurement of the molecular ion. On the basis of the HRMS experiments, the elemental composition of TriBHD was unequivocally established to be $C_{16}H_{19}Br_3O$.

Search of the literature brought only three known compounds with this molecular formula to our attention. The first one (i) was an intermediate synthesis product with a tribromophenyl unit (*19*), the second one (ii) was a substituted tetrahydronaphthalene with a carboxytribromomethyl substituent (*20*), whereas the third one (iii) was a hexahydroxanthene derivative isolated from sponges (*21*). Variants (i) and (ii) could be ruled out because of the structural features mentioned above, which are linked to distinct mass spectrometric fragmentations not found in the GC/EI-MS of TriBHD. By contrast, the mass spectrum of TriBHD was virtually identical with data published for (iii) by Garson and co-workers (*21*). Moreover, Garson et al. showed that *m/z* 185 {$[C_7H_6BrO]^+$ (*21*)} and the nonbrominated base peak at *m/z* 121 are most likely formed from *m/z* 306 (*21*). It seems that *m/z* 121 represents a dimethylmethylene cyclohexadienyl cation ($[C_9H_{13}]^+$). This was confirmed in consecutive HRMS measurements where the measured mass of *m/z* 121.1016 matched the theoretical value of *m/z* 121.1017 with high precision. In addition, the HRMS data provided by Garson et al. were identical with ours, and finally, the presence of a tetrabromo congener (TetraBHD) was only mentioned in this paper (*21*). In contrast to TriBHD, TetraBHD showed an abundant molecular ion in GC/ECNI-MS at *m/z* 542 (**Figure 5**). Fragment ions were found at *m/z* 464/465 (three Br), *m/z* 384 (two Br), and the bromide ion (*m/z* 79). Likewise, the molecular ion was detected in GC/EI-MS mode, and the tribromo fragment ion at *m/z* 384 and dibromo fragment ion at *m/z* 264 correspond with *m/z* 306 and *m/z* 185 in the GC/EI-MS of TriBHD.

The mass spectrometric similarity along with the coappearance of both compounds in fish verified that both compounds belong to the same substance class, and we conclude that TriBHD and TetraBHD are identical with the two polybrominated hexahydroxanthene derivatives (PBHDs) determined in sponges of the *Cacospongia* genus (**Figure 6**) (*21*). The PBHDs were initially identified in sponges from Australia, but *Cacospongia* were reported to occur in the Mediterranean Sea as

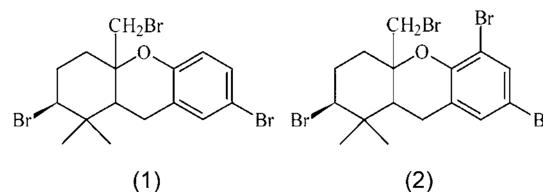

Figure 6. Structures of polybrominated cacoxanthenes TriBHD and TetraBHD according to ref *21*.

well. While there is clear evidence for the hexahydroxanthene backbone of the PBHDs, it cannot be ruled out that the bromine substituents are found at different positions than those shown in **Figure 6**. However, the final proof would have required higher amounts for ^{1}H NMR studies.

Concentrations of TriBHD and Other HNPs. A wide range of fish was investigated for PBHDs and other HNPs (TBA, Q1, and MHC-1) as well as for *p,p′*-DDE (**Table 1**). Because of the initial detection of PBHDs in gilt head bream (*Sparus aurata*) from the Mediterranean Sea, we first focused on this and related species. Gilt head bream and sea bass (*Labrax lupus*) are "fashion" fish, which are sold in increasing amounts in Germany. For example, they are very frequently served in Italian style restaurants. The reason for the steadily growing market is fish aquacultures in the Mediterranean Sea similarly to salmon farms in the Atlantic. Note that fish from aquacultures usually contain higher lipid contents than free-living individuals. Of the 14 samples investigated, TriBHD and TetraBHD were detected in 11 samples. Interestingly, two of the negative samples were from wild fish while all cultivated samples except one contained the PBHDs. Usually, TriBHD was about seven times more abundant than TetraBHD. TBA, MHC-1, and Q1 were detected in 13, 11, and 12 samples, respectively. Usually, *p,p′*-DDE caused the most abundant peak in the fish samples (exception: one sample, which was dominated by Q1 and one by TBA) but TriBHD could account for more than 90% of the *p,p′*-DDE content (**Table 1**).

PBHDs were also detected in two of five samples of sardine and anchovy. Furthermore, 10 out of 19 samples of salmon (*Salmo salar*) from aquacultures contained low concentrations of TriBHD (TetraBHD was below the detection limit) but eight samples of wild salmon (*Oncorhynchus keta*) did not contain PBHDs (data not shown). Farmed salmon contained high concentrations of TBA and MHC-1, which were particularly abundant in the samples from the Faeroe Islands. The concentration of 934 ng/g lipid weight matches the highest concentration for MHC-1 determined to date in fish (*6*). In addition, 26 salmon oil capsules and four ω-3-fish oil capsules (species of fish not mentioned) were analyzed for PBHDs. TriBHD was present as low abundant peaks in 17 samples (1−5 ng/g fish oil), whereas TetraBHD was below the detection limit. Note that the fish oils are usually refined in order to reduce their content of halogenated pollutants. We also detected the PBHDs in a monk seal sample from Mauritania, which indicated that this substance class may reach the end-links of food webs and thus also humans.

Because of the initial determination in sponges from Oceania, we also reinspected samples of green-lipped mussels from New Zealand for PBHDs. High concentrations were determined in both samples (**Table 1**). Even more surprising, the highest concentration of PBHDs (>200 ng/g l.w.) was determined in this species, and both PBHDs were also detected in powdered mussels sold in gelatin capsules as nutritional supplements. While *p,p′*-DDE was virtually absent in the green-lipped mussel

Table 1. Concentrations (ng/g Lipid Weight) of TriBHD, TetraBHD, TBA, MHC-1, Q1, and *p,p*′-DDE in Commercial Fish and Mussels

species	zoological name	farmed/free	origin	lipid content (%)	concentrations in ng/g lipids[a] TriBHD	TetraBHD	TBA	MHC-1	Q1	*p,p*′-DDE
			sea bass and gilt head bream (2005)							
sea bass	*L. lupus*	aquaculture	Italy	11.6	59	10	11	ND	5	66
sea bass	*L. lupus*	aquaculture	Italy	3.5	66	9	14	14	11	110
sea bass	*L. lupus*	aquaculture	unknown	10.1	90	13	12	17	ND	139
sea bass	*L. lupus*	aquaculture	unknown	7.2	22	3	6	6	11	88
sea bass	*L. lupus*	aquaculture	unknown	2.3	ND	ND	22	52	217	65
gilt head bream	*S. aurata*	aquaculture	Greece	13.9	6	1	37	1	3	12
gilt head bream	*S. aurata*	aquaculture	Greece	8.1	11	1	16	1	5	47
gilt head bream	*S. aurata*	aquaculture	Greece	10.5	19	3	12	1	9	51
gilt head bream	*S. aurata*	aquaculture	Greece	7.3	19	3	14	1	ND	55
gilt head bream	*S. aurata*	aquaculture	Greece	11.3	32	4	12	1	4	52
gilt head bream	*S. aurata*	aquaculture	France	10.2	16	2	19	1	6	56
gilt head bream	*S. aurata*	aquaculture	unknown	8.8	16	2	22	1	15	65
gilt head bream	*S. aurata*	free living	Pacific	0.9	ND	ND	ND	ND	22	33
gilt head bream	*S. aurata*	free living	middle East Atlantic	4.4	ND	ND	2	ND	86	11
			sardine and anchovy (2001)							
sardine	*Sardina pilchardus*		Italy	3.2	59	ND	3	3	ND	375
anchovy	*Anchoa* spp.		unknown	8.6	23	ND	29	2	ND	209
			salmon (2004)							
salmon	*S. salar*	aquaculture	Faeroe Islands	16.0	3	ND	17	938		
salmon	*S. salar*	aquaculture	Ireland	16.7	1	ND	90	13		
salmon	*S. salar*	aquaculture	Norway	16.1	2	ND	24	19		
salmon	*S. salar*	aquaculture	Norway	23.1	4	ND	10	30		
salmon	*S. salar*	aquaculture	Norway	16.7	1	ND	5	10		
salmon	*S. salar*	aquaculture	Norway	13.6	1	ND	39	14		
salmon	*S. salar*	aquaculture	Norway	17.1	2	ND	29	15		
salmon	*S. salar*	aquaculture	Norway	19.9	2	ND	24	19		
salmon	*S. salar*	aquaculture	Norway	15.3	1	ND	10	10		
salmon	*S. salar*	aquaculture	Norway	22.1	ND	ND	6	10		
			green-lipped mussels (2004)							
green-lipped mussel	*P. canaliculus*	aquaculture	Neuseeland	3.9	144	92	123	ND	ND	ND
green-lipped mussel	*P. canaliculus*	aquaculture	Neuseeland	4.0	25	8	250	ND	ND	5

[a] ND, not detected; no value, not determined.

samples, PBHD concentrations and also those of TBA exceeded the residue limits of some chloropesticides in seafood.

Relevance and Ecological Role of TriBHDs. As was shown above, the highest PBHD concentrations were determined in samples from aquacultures. While there is a chance for uptake via commercial food, we suggest that the bulk of HNPs originated from local sources. Fish farms are located close to the seashore where the potential natural producers, sponges of the *Cacospongia* genus, have their habitat. The strong variation in the PBHD concentrations along with differences in the concentrations relative to *p,p*′-DDE supports this theory. Given the high lipid content of fish from aquacultures (see **Table 1**), the problem of contamination of fish with HNPs is becoming more relevant (*22*). HNPs have diverse chemical functions in living organisms ranging from chemical defense over food gathering to hormone regulation (*12*). When looking at the structure of the PBHDs, the inclusion of a bromophenol unit is conspicuous (**Figure 6**, right part of the structure). Simple bromophenols and -anisoles are known secondary metabolites of algae, marine worms, and other organisms (*12*, *23*, *24*). Therefore, it could be possible that the bromophenols serve as substrates for the building up of larger molecules. In this context, the relatively new insight of bromine being a good protection/leaving group in aromatic compounds deserves discussion (*25*).

The steadily growing prevalence of HNPs in marine food may be due to nontraditional hunting places (e.g., samples from the southern hemisphere) and the installation of farms along the coastline without a long tradition in fishing. The higher lipid content of cultivated fish will be another reason for the higher abundance of lipophilic HNPs. We thus conclude that there is a need for a more detailed research on the occurrence, distribution, and potential toxic effects of HNPs in seafood.

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